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INFLUENCE OF DROUGHT ON THE CONCENTRATION AND DISTRIBUTION OF 2,4-DIAMINOBUTYRIC ACID AND OTHER FREE AMINO ACIDS IN TISSUES OF FLATPEA (*LATHYRUS SYLVESTRIS* L.)

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SHEN L., ORCUTT D. M. and FOSTER J. G. *Influence of drought on the concentration and distribution of 2,4-diaminobutyric acid and other free amino acids in tissues of flatpea* (Lathyrus sylvestris *L.*). ENVIRONMENTAL AND EXPERIMENTAL BOTANY **30,** 497–504, 1990.—2,4-Diaminobutyric acid (A_2bu) may be responsible for the apparent toxicity of flatpea (*Lathyrus sylvestris* L.) forage to some livestock. To obtain information relative to environmental regulation of A_2bu, 3-month old flatpea plants, cv. "Lathco", were subjected to water-deficit stress for 1, 2, and 4 weeks. A_2bu, the most abundant free amino acid in roots, stems, and leaves, increased nearly 100% in roots of stressed plants. Increases in the concentrations of asparagine (Asn), proline (Pro), and arginine (Arg) occurred in roots; Asn, Pro, and 4-aminobutyric acid (Abu) in stems; and Pro and homoserine (Hse) in leaves also occurred in response to drought stress. Proline was a minor constituent of the free amino acid pool, even under water-deficit stress. The distribution of A_2bu and Pro in the stressed plants (roots > stems > leaves) was the reverse of that in plants supplied with adequate water (roots < stems < leaves). As concentrations of Asn and Abu decreased from roots to leaves in control tissues, concentrations of Hse and A_2bu increased in roughly the same proportions. This observation suggests that Abu and Asn may be precursors of A_2bu and Hse, respectively. The increase in A_2bu levels in aerial parts of drought-stressed flatpea plants is probably not sufficient to lower the feed value of the forage.

Key words: 4-Aminobutyric acid, asparagine, 2,4-diaminobutyric acid, homoserine, drought, proline, flatpea.

INTRODUCTION

FLATPEA (*Lathyrus sylvestris* L.) is a long-lived, deep-rooted, perennial legume that is tolerant to environmental stresses such as drought,[28] low soil fertility,[1] low temperature,[25] and low soil pH.[18] Because of these characteristics, flatpea is currently under study as a potential forage species for use in northern temperate regions where environmental stresses limit the growth and productivity of other legumes.[16,28] The presence of A_2bu in this plant and the influence of environmental stresses on its concentration have been sources of concern because of its neurotoxic potential in animals.[10,23]

Drought-stress effects on plant nitrogen metabolism have been well documented. Major changes include enhanced proteolysis and inhibition of

protein synthesis.[2,7,13,21,26] Associated with these effects is the accumulation and/or depletion of protein- and non-protein-amino acids. The accumulation of Pro in drought-stressed plants has been of considerable interest to plant scientists because it appears to respond more profoundly than do most other amino acids.[12,17,20] Little attention has been given to other nitrogenous compounds which may also accumulate in plants and contribute to drought tolerance.[29] Asparagine and Abu have been reported to increase in water-stressed wheat plants,[7] citrus seedlings,[6] and cotton leaves.[17] In wheat, the response differed with cultivar, organ, and stage of development. Asparagine also increased in water-stressed Bermuda grass shoots.[2]

The purpose of the present study was to quantify A_2bu and other protein- and non-protein-amino acids in leaf, stem, and root tissues of flatpea plants grown under different levels of drought in an effort to obtain information concerning changes in A_2bu levels and distribution in the plant and to gain insight into the physiological function of A_2bu.

MATERIALS AND METHODS

A typical Appalachian top-soil, Lily (fine-loamy, siliceous, mesic Typic Hapludult), was used to grow experimental plants. Soil pH was 5.5 and was supplemented with K_2HPO_4 at a rate of 0.5 g/kg of dry soil. The soil was covered with a heavy plastic sheet and sterilized with methylbromide (50 g per 1 kg of soil, Dow Chemical Co., Midland, MI) for 2 days. Soil moisture retention curves were determined using a pressure plate extractor (Soil Moisture Equipment Co., Santa Barbara, CA).

Flatpea (*Lathyrus sylvestris* L. cv. "Lathco") seeds were scarified with concentrated H_2SO_4 for 10 min and imbibed in continuously aerated water for 2 days at 20°C in the dark. After imbibition, each seed was inoculated with 50 ml of a late-log phase *Rhizobium leguminosarum* culture (10^9 cells/ml), strain 92F2 (Nitragin Co., Milwaukee, WI). Inoculated seeds were sown in 20 cm plastic pots (10 seeds per pot) containing 3.5 kg of dry soil. Experiments were conducted in controlled environment growth chambers with a photoperiod of 16 hr. An average photosynthetic photon flux density of 400 μmol m^{-2} sec^{-1} (400–700 nm) at plant height was provided by a combination of fluorescent (215 W cool white high output 96T12 lamps, Philips Lighting Corporation, Bloomfield, NJ) and incandescent (Sylvania 60 W, inside frosted, GTE Corp., Springfield, VA) lamps. Light/dark temperatures were 27/20°C and the relative humidity was 75%. Soil water potential was maintained as close to −0.1 MPa as possible by weighing and adding an appropriate amount of water to each pot daily. One week after emergence, plants were thinned to four plants per pot. At 40 days post-emergence, shoots were pruned to the soil surface. After 40 days of regrowth the pots were divided into two groups. Soil water potential of the control group was maintained at approximately −0.1 MPa. Moisture was withheld from plants in the second group until the soil water potential reached −1.2 MPa (approximately 2–3 days), after which water was added daily, as needed, to maintain this potential. One, 2, and 4 weeks after initiation of the stress three pots each of water-stressed and control plants were harvested. Water potentials of leaves from the two treatments were measured with a pressure bomb (Soil Moisture Equipment Corp., Santa Barbara, CA). Harvested plants were separated into leaves, stems, and roots. Tissues were crushed in liquid nitrogen with a mortar and pestle and a portion of the fresh, frozen tissue was used for soluble protein analysis. Five milliliters of 0.1 M HEPES-NaOH (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5; Calbiochem-Behring Corp., La Jolla, CA), containing 0.01 M isoascorbate (Sigma Chemical Co., St. Louis, MO) and 0.05% (v/v) antifoam C emulsion (Sigma Chemical Co.), was added to 0.5 g of insoluble polyvinylpyrrolidone (Sigma Chemical Co.) and 1 g of fresh, frozen tissue. The mixture was homogenized in an ice bath using a Polytron homogenizer (Brinkman Model PT 10-35, Westbury, NY) with a Model PT 10-ST probe generator at maximum speed for 20 sec. Extracts were centrifuged at 10,000 ***g*** for 20 min in a Beckman (Palo Alto, CA) model JA-20 rotor. The resulting pellet was resuspended in 5 ml of the initial homogenizing medium and centrifuged as before. Supernatants from the two centrifugations were

combined and soluble protein was assayed using the procedure of BRADFORD.[5]

The remaining fresh, frozen tissue was lyophilized (Virtis 10-100-V, Gardinar, NY) to constant weight and stored at −20°C for other analyses. Total nitrogen content of lyophilized tissue was measured using a Leco (St. Joseph, MI) model CHN-600 carbon–hydrogen–nitrogen analyzer. Amino acids were extracted and analyzed as outlined previously.[27] Briefly, approximately 0.5 g of lyophilized tissue was extracted with 90 ml of 50% (v/v) aqueous ethanol for 90 min using Soxhlet extractors. Two milliliters of 15 mM *S*-(4-pyridylethyl)-DL-penicillamine (Pierce Chemical Co., Rockford, IL) was added as an internal standard. Sample extracts were concentrated under nitrogen at 40°C and the resulting residue was resuspended in 10 ml of extraction medium. A 2.5 ml aliquot of this suspension was centrifuged and the pellet was resuspended in 2.5 ml of extraction medium and recentrifuged twice. The three supernatants were combined and brought to a total volume of 7.5 ml with extraction medium. An extract volume of 0.25 ml was loaded onto a Sep-Pak C_{18} column (Waters Associates, Milford, MA) and eluted successively with 0.5 ml of water and 1.0 ml of methanol. The combined eluate was brought to 2.0 ml with water. Twenty milliliters of eluent was reacted with 0.1 ml of *o*-phthalaldehyde for 90 sec and then injected into a 45 mm, 5 μm Ultrasphere-ODS precolumn and an Altex 4.6 × 250 mm, 5 μm Ultrasphere-ODS analytical column maintained at 45°C. Amino acid derivatives were detected using fluorescence detection (305–395 nm excitation and 430–470 nm emission). Amino acids were identified by comparing their retention times to those of pure amino acid standards and by coinjection of known amino acids. Amino acids were quantified using standard curves for each amino acid generated over the concentration range found to occur in the tissues examined. Yields of individual amino acids in extracts were adjusted based on recoveries of the internal standard. Proline concentrations were determined using the acid-ninhydrin method of Ref. 3. Each treatment consisted of three replications (four plants per replication) arranged in a randomized complete block design. All growth and chemical measurements were performed on the three replications. Standard error of the means was determined for each treatment as was analysis of variance at the 0.05 level of significance.

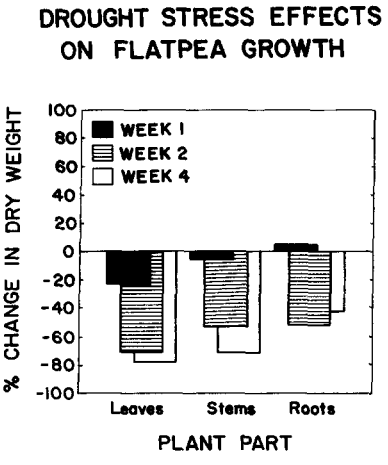

FIG. 1. Per cent change in dry weight of flatpea leaves, stems and roots at the end of a 4-week drought period.

RESULTS

Leaf water potentials of control and water-stressed plants ranged from −0.6 to −0.8 MPa and −1.5 to −1.8 MPa, respectively, over the duration of the experiment. A water potential difference of −0.7 to −0.9 MPa was observed between the controls and stressed plants throughout the test period, with a maximum standard error of −0.19 MPa.

Relative to controls, total plant biomass decreased in the stressed plants over the 4-week period (Fig. 1). Leaf growth was inhibited more than stem or root growth and the longer the duration of the stress the greater the growth inhibiton of the leaves and stems. Root dry matter increased slightly during the first week of stress, but this effect was offset by a decrease in leaf and stem dry weight. Plants exposed to a water potential of −1.2 MPa for 2 weeks exhibited root dry weights significantly ($P \leqslant 0.05$) less than the controls.

Total nitrogen was highest in the leaves while the stems and roots had similar concentrations

(Fig. 2). In all tissues of the control plants nitrogen concentration changed little from week 1 to week 4, but a trend towards reduced nitrogen was observed in roots of control plants as the experiment progressed. Water-deficit stress did not affect the total nitrogen concentration in the leaves or stems of flatpea. However, total nitrogen increased in the roots by 42 and 46% after 2 and 4 weeks of drought stress, respectively (significant at $P \leqslant 0.05$ for both weeks).

The highest concentration of soluble protein was observed in the leaves followed by the stems and roots of control flatpea plants (Fig. 3). Soluble protein tended to increase in all tissues of stressed and control plants (exception, stem control) with age. Leaf tissues of stressed plants were lower in soluble protein than control plants at all stress periods (week 2 significant at $P \leqslant 0.05$). In the stems and roots of 1-week stressed plants soluble protein was lower than in the controls (roots significant at $P \leqslant 0.05$), but

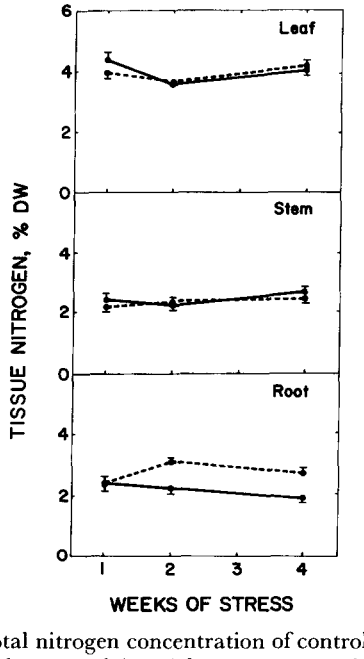

FIG. 2. Total nitrogen concentration of control (——) and drought-stressed (- - - -) leaves, stems and roots of flatpea plants over a 4-week stress period. Error bars represent the standard error of three replications. Data without confidence intervals are less than one standard deviation.

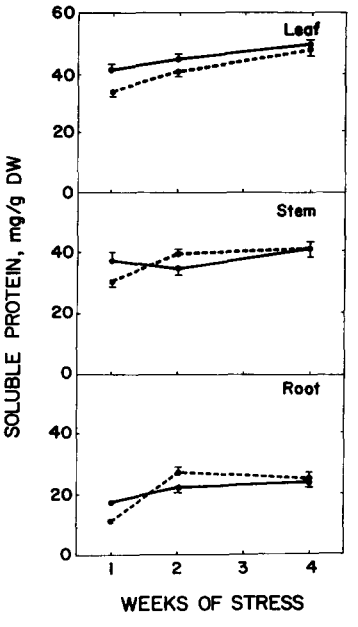

FIG. 3. Soluble protein concentrations of control (——) and drought-stressed (- - - -) flatpea leaves, stems and roots over a 4-week stress period. Error bars represent the standard error for three replications. Data without confidence intervals are less than one standard error unit.

these levels surpassed the controls after 2 weeks. After 4 weeks the soluble protein concentrations were similar in both stems and roots and were not significantly different.

The major free amino acids detected in the leaves, stems, and roots of flatpea control tissues were Abu, A_2bu, Asn, Hse, and Pro (Fig. 4). Alanine, aspartate, glutamine, glutamate, isoleucine, leucine, phenylalanine, serine, and valine were also detected at levels of less than 1 mg/g dry weight (data not shown). Control leaves were characterized by having high concentrations of the following amino acids in the indicated relative abundance: A_2bu > Hse > Abu > Pro (exception, at week 4 Asn higher than Pro). In the control stems the relative abundance of the major amino acids was: A_2bu > Hse (except week 1) > Abu > Asn. Relative concentrations in the control roots were: A_2bu (except week 1) > Abu > Asn (except week 1) > Hse and Asp, the latter two being similar in concentration. It

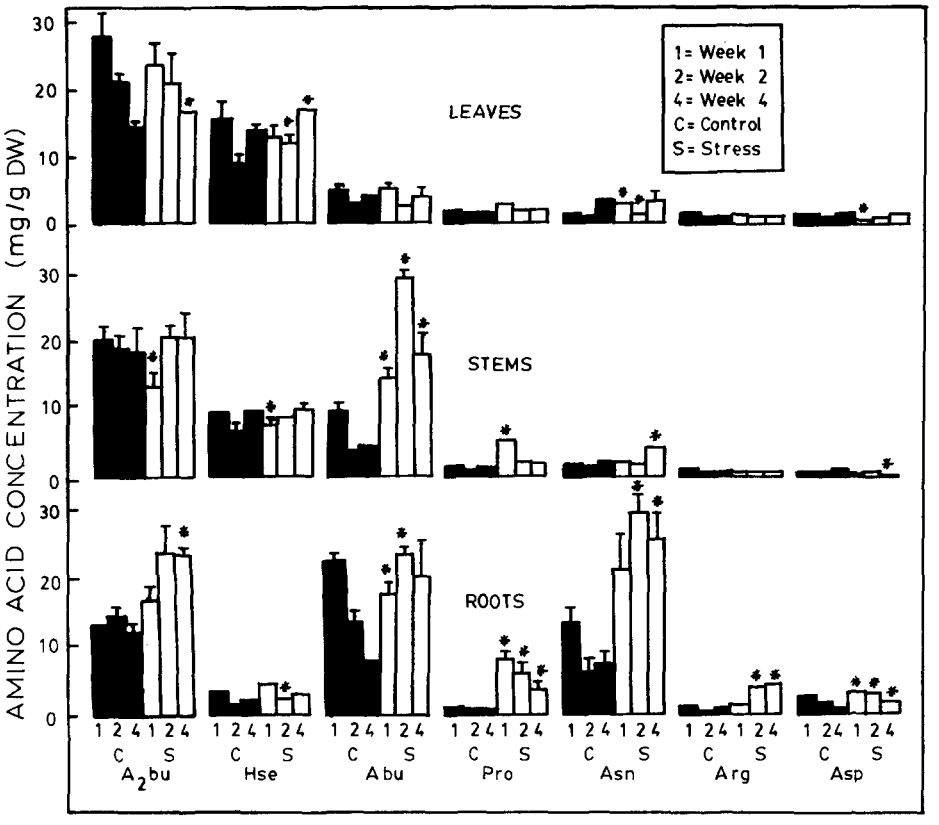

FIG. 4. Amino acid composition of flatpea plants subjected to water-deficit stress for 1, 2, and 4 weeks. Bars without confidence intervals are less than one standard error unit. * Indicates significant difference compared to the respective control at $P \leqslant 0.05$.

also appears than an inverse relationship exists between Asn and Hse and between Abu and A_2bu. As Asn and Abu decreased in concentration from roots to leaves, Hse and A_2bu increased in roughly the same proportions.

Water-deficit stress caused the largest change in total free amino acid concentration in the roots, where an increase of 30, 146 and 156% was observed after 1, 2, and 4 weeks of stress, respectively. Stems exhibited the next highest increase (week 2, 104%; week 4, 52%). Stress-related changes in free amino acid levels were the smallest (week 2, 14%; week 4, 23%) in leaves. Changes observed in the stems and roots were significant ($P \leqslant 0.05$) but not the leaves.

Accumulation of several amino acids occurred in flatpea plants subjected to drought stress (Fig. 4). Pro accumulated in all tissues, the greatest change occurring at week 1 and declining thereafter. In the control plants the highest concentrations of Pro were in the order of leaves > stems > roots. When the plants were stressed the order was reversed. Asparagine was particularly prevalent in the roots and increased with the duration of the stress to a level three to four times that in roots of control plants. As the duration of the stress increased Arg was also observed to accumulate in the roots, but to a much lower extent than Asn. In stems, Abu increased when plants were subjected to water stress, but decreased during the later part of the experiment (week 4). Imposition of water stress did not reverse the relative

distribution of Asn, Abu, or Hse among the roots, stems, and leaves from those observed for control tissues (Asn, Abu: roots > stems > leaves; Hse: roots < stems < leaves). Levels of the previously mentioned minor amino acid constituents remained relatively constant in all tissues throughout the stress period.

Although total amino acid concentration increased under the stress conditions employed, levels of some amino acids in the leaves (Asn-week 2 and Asp-week 1) and stems (A_2bu and Hse-week 1, Asp-week 4) declined. The only amino acid to significantly decline in the roots was Abu (week 1).

Of particular interest to us in this study were the high concentrations of A_2bu in the plant tissues and the changes that occurred as a result of water stress. Little difference was observed in this amino acid between stressed and control plants in leaves or stems (Fig. 4). However, root levels of A_2bu increased significantly in stressed plants by nearly 100% compared to the controls at week 4. While control levels of A_2bu in roots were consistently less than those in stems and leaves water stress tended to have an equalizing effect on the concentration of A_2bu in all tissues. A_2bu decreased with age in control and stressed leaves but increased in stressed stems and roots of the second and fourth weeks compared to the first week. In general, the amino acid concentration in most control plants decreased with age in all tissues. This was particularly true when weeks 2 and 4 were compared to week 1 (exceptions week 2 A_2bu in roots and Asn in week 4 leaves). Conversely, stressed plants frequently exhibited increases in amino acids, particularly in stems and roots, with age (exceptions, Pro in all tissues, Asn in stems, and Asp in roots).

DISCUSSION

Changes observed in soluble protein, free amino acid, and total nitrogen levels of several drought-stressed plant species have been attributed to a reduction in the rates of protein synthesis and an increase in proteolytic activity,[2,6,11,30] both of which tend to cause an increase in total soluble nitrogen. The accumulation of organic solutes such as free amino acids may also allow plants to overcome water stress through osmotic adjustment[9] and/or serve as storage forms of nitrogen and carbon for future use under less stressful conditions.[2] In flatpea, several free protein- and non-protein-amino acids responded to drought stress by either decreasing or increasing in concentration, but the particular response depended on the amino acid, tissue analyzed, and the duration of the stress (Fig. 4). Differences in amino acid composition between cultivars, organs, and developmental stages of wheat and flatpea plants subjected to drought stress have been reported.[7,27]

Plants have been classified into species which do or do not accumulate free proline under water stress.[20] For example, in stressed cotton leaves only 10% of the total free amino acids was Pro,[29] while in stressed Coastal Bermuda grass, Pro comprised 42% of the total.[2] Drought-tolerant *Artemisia herba-alba* had a low level of free Pro in the non-stressed state and did not accumulate Pro when drought stressed.[22] Although Pro increased in drought-stressed flatpea plants (Fig. 4), Pro comprised only 10–11% of the total concentration of free amino acids in the stressed plants.

A function of Pro in osmo-adjustment has been proposed[15] although there may be no cause and effect relationship between Pro accumulation and osmo-regulation in plants under water-deficit stress[29] as suggested by differences in Pro concentrations and responses of plant species to drought.

In addition to Pro, several other amino acids, including Asn, Arg, Abu, and A_2bu in the roots, Abu and Asn in the stems, and Abu, A_2bu, and Hse in the leaves, increased in flatpea in response to the water-deficit stress. These amino acids contributed much more to the total plant nitrogen than did Pro. Accumulation of Asn in water-stressed plants has been reported previously.[2,6,7,17,30] While the role of Asn as an important nitrogen transport compound in plants has been well established,[14] the specific role of Abu is still unclear. Increases in Abu in response to water stress have been observed in cotton leaves[17] and wheat plants,[7] and CHEN *et al.*[6] reported an increase in the Abu content of leaves and roots of citrus seedlings that generally occurred at the wilting range of water stress. Why flatpea plants accumulate Abu in stems when stressed is not clear.

The apparent inverse relationship between Asn and Hse and between Abu and A_2bu in the leaves, stems, and roots of flatpea may suggest biosynthetic relationships. Comparisons of amino acid levels in roots and leaves revealed that the magnitude of the decrease in concentration from one plant part to the other for one member of each pair of amino acids was generally similar or equal to the amount of increase of the other member of the amino acid pair for the corresponding tissues (Fig. 4). It is possible that Abu and Asn are precursors to A_2bu and Hse, respectively, the former two amino acids being synthesized in the roots and transported to the stems and leaves where A_2bu and Hse are synthesized. Intracellular localization studies[8] support the hypothesis that A_2bu synthesis occurs in the leaves, and elevated levels of A_2bu in root tissue of drought-stressed plants could result from transport of this amino acid from the leaves. Further studies are required to determine if A_2bu and Abu are synthesized exclusively in the leaves and/or roots, respectively. Evidence in the literature for the synthesis of A_2bu from Abu is lacking, but studies with pea shoots grown initially on $[^{15}N]O_3^-$ resulted in 80% of the amide N of Asn being metabolized in 2 hr, and the amino acid receiving the labeled N most rapidly was Hse.[4] Studies in which cut flatpea seedlings were provided with either L-[^{3}H] homoserine or DL-[1-^{14}C]asparatic acid resulted in incorporation of the radioactive label into free A_2bu.[19] These results suggested that both Asp and Hse can serve as precursors for A_2bu. Asn and Asp are readily interconverted enzymatically. The most abundant amino acid in flatpea tissues was A_2bu. This was true for non-stressed and stressed plants with only a few exceptions where Abu was slightly higher in the stems and roots. While nearly a 100% increase in the concentration of A_2bu was observed in the roots of stressed plants, the concentration of A_2bu in the leaves and stems did not respond to water stress as dramatically as several of the other amino acids. The increase in A_2bu concentration cannot be directly related to protein degradation since A_2bu is a non-protein amino acid and the soluble protein concentration was unaffected (Fig. 2). It is conceivable that A_2bu accumulation in flatpea roots functions in osmo-regulation or nitrogen storage for use upon removal of the stress. Considering the low toxicity of A_2bu[24] and the quantity of A_2bu in leaf and stem tissues from control plants at week 1 and those in corresponding tissues from all stressed plants (Fig. 4), we doubt that the marginal increases observed in the concentrations of A_2bu in the leaves and stems would diminish the quality of flatpea forage, but no information is available regarding acceptable levels of A_2bu in forage used for livestock feed. Livestock feeding studies using flatpea will be the focus of future studies that will address this problem.

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